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Biotechnological production of zeaxanthin by an Antarctic *Flavobacterium*: Evaluation of culture conditions

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Highlights

- An Antarctic *Flavobacterium* sp. was evaluated as a source of zeaxanthin.
- Effects of temperature and nutritional factors were studied.
- Oxygen was found to be an important factor for improving zeaxanthin yield.

Abstract

Statistical experimental designs were used to formulate a culture medium for zeaxanthin production by an Antarctic *Flavobacterium* sp. P8 strain. Eleven nutritional factors were assayed in shaken flasks. The effect of temperature on zeaxanthin and carotenoid production was also studied. Peptone, yeast extract, and sodium chloride were the nutrients that caused the principal impact on the biomass growth. These components were further studied to enhance zeaxanthin and total carotenoid concentrations. Although a high production rate of zeaxanthin and carotenoids was achieved, the aerobic characteristics of the bacterial strain and the oxygen requirements for zeaxanthin biosynthesis incorporate a factor that requires additional consideration. Scaling up the process to a 5 L-bioreactor that increased dissolved oxygen availability resulted in a 4.5-fold increase in the total carotenoid content and an almost 9-fold

increase in zeaxanthin, which represented 98% of the total carotenoids produced. The results reveal that *Flavobacterium* sp. P8 is a promising strain for zeaxanthin production.

Keywords

Carotenoids, Zeaxanthin, *Flavobacterium*, experimental design, bioreactor

1. Introduction

Zeaxanthin (3,3´-dihydroxy-β-carotene) is an oxygenated carotenoid (xanthophyll) that performs important physiological functions (George Britton, 1995). Although it has no pro-vitamin A activity, zeaxanthin is beneficial for human health, such as in the prevention of macular degeneration. Carotenoids' ability to decrease the risk of some types of cancer due to their ability to extinguish free radicals, exert antioxidant effects, and reduce inflammation has been studied (Krinsky et al., 2003). Like other animals, humans are unable to synthesize carotenoids, which are, therefore, obtained from dietary sources (Delgado-Pelayo and Hornero-Méndez, 2012). Zeaxanthin is highly valued in animal feed because it enhances the pigmentation of meat and eggs (Shin et al., 2016).

Carotenoids are obtained industrially by chemical synthesis or extraction from plants and algae (Navarrete-Bolaños et al., 2005; Valduga et al., 2009). In particular, zeaxanthin can be found in microorganisms, higher plants and green algae. Zeaxanthin and lutein can be extracted from marigold flowers by lixiviation with hexane. After a concentration stage and further purification, a mixture of xanthophylls suitable for human consumption is produced (Navarrete-Bolaños et al., 2005). Zeaxanthin can also be produced by chemical synthesis through the Wittig's reaction. However, this process involves numerous reaction steps, and the product has poor biological activity since the three possible stereoisomers 3R,3'R-, 3S,3'S-, and 3R,3S- are obtained along with some zeaxanthin degradation molecules (Sajilata et al., 2008).

The production of zeaxanthin and other carotenoids by microbial sources is a field of increasing interest since consumers are shifting to the use of natural and environmentally friendly products. In contrast to other carotenoids, such as β-carotene, astaxanthin, and lycopene, which are produced by a wide variety of microorganisms, only a few bacteria, cyanobacteria, and microalgae synthesize zeaxanthin. Some bacterial genera, such as *Flavobacterium, Erwinia, Formosa,* and *Muricauda*, have been reported to produce zeaxanthin (Zhang et al., 2018). The production of zeaxanthin in bioreactors has some advantages over traditional methods. It offers the possibility of using low-cost substrates, is independent of climate dependency, and does not compete with land for growing food.

In addition, zeaxanthin purification using a bacterial source requires a less demanding process, in comparison to algae or plants. Bacteria mainly produce zeaxanthin, and generally lower amounts of β-carotene and β-cryptoxanthin. In contrast, frequently algae and plants produce together with zeaxanthin, other carotenoids such as lutein, which is a structural isomer much more difficult to separate. Besides, microorganisms produce the natural stereoisomer (3R,3'R)-zeaxanthin, which presents the highest biological activity. Bacteria, such as *Flavobacterium multivorum*, produce almost exclusively (3R,3'R)-zeaxanthin in its free form (Sajilata et al., 2008).

Statistical experimental designs have been widely used in research on improving and optimizing pigment production, as well as evaluating carbon and nitrogen sources, salts, and vitamins and metabolic enhancers to promote carotenoid accumulation (Bhosale et al., 2004; Liu and Wu, 2007; Masetto et al., 2001). The formulation of culture media optimizing production yields is of great importance to developing an economically competitive bioprocess.

Another relevant aspect of microbial zeaxanthin production is oxygen availability. Oxygen is reportedly needed to hydroxylate β-carotene and β-cryptoxanthin during zeaxanthin production (G Britton, 1995; Masetto et al., 2001). Masetto (2001) studied the *Flavobacterium* strain ATCC 21588 in a 4-L fermenter and showed that with an adequate amount of nutrients, as well as a high agitation speed and aeration, zeaxanthin production was improved.

The aim of this work was to study the potential for zeaxanthin production by *Flavobacterium* sp. P8 isolated previous at Maritime Antarctica (Vila et al., 2019). A medium culture formulation was optimized for zeaxanthin and total carotenoid production in shaken flasks and a bioreactor.

2. Materials and methods

2.1 Microorganisms and culture conditions

Flavobacterium sp. P8 strain was isolated from saltwater samples collected at King George Island in Antarctica during an expedition organized by the *Instituto Antártico Uruguayo* [Uruguayan Antarctic Institute] in December 2014 (Vila et al., 2019). Stock cultures were maintained in 20% glycerol and stored at -80ºC.

2.2 Inoculum preparation and flask fermentation

Biomass used for inoculation was grown in 500 mL-Erlenmeyer flasks with 150 mL of Tryptic Soy Broth (Merck) supplemented with 1% Sea Salt (Sigma) for 24 h at 20ºC in an orbital shaker at 200 rpm.

Fermentations were carried out in 1 L-Erlenmeyer flasks with 300 mL of medium culture at 20ºC and 200 rpm. The initial pH was adjusted to 7.0 with NaOH and media inoculated with 10% v/v of an exponential phase culture. Biomass growth was monitored by measuring the optical density of the culture at 600 nm (OD_{600}). After 48 h, cells were harvested by centrifugation 15 min at 6500 rpm and 4°C (Thermo Scientific, IEC CL30R). Pellets were washed with distilled water, frozen at -80ºC, and lyophilized (VirTis BenchTop 2 K Freeze Dryer, SP Industries Inc.).

2.3 Temperature effects on cell growth, zeaxanthin production, and total carotenoid production

Cultures in a basal medium were grown in duplicate at 15, 20, 25, and 30ºC in an orbital shaker, as described in Section 2.2. The basal medium composition was 6 g/L peptone, 2 g/L yeast extract, and 20 g/L sea salt, and the pH was adjusted to 7. Biomass was monitored by optical density, and in the early stationary phase, cultures were harvested for carotenoid quantification, as described in Section 2.6.

2.4 Experimental design and data analysis

A Plackett-Burman design was used to screen the effects of 11 nutritional factors of the culture medium and determine their impact on biomass production. Four dummy variables were added to estimate response error, resulting in a total of 16 trials. For each variable, two levels were tested: high concentration (+) and low concentration (-). Carbon and nitrogen sources, as well as mineral salts, were selected based on previous assays (data not shown). The composition of the micronutrient solution was H_3BO_3 (12.8 g/L), $LiSO_4$ (1.0 g/L), $MnSO_4$ (3.2 g/L), $CoCl_2$ (2.0 g/L), $CuSO_4 \cdot H_2O$ (4.0 g/L), $NiSO_4$ (2.5 g/L), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (2.8 g/L), and $ZnSO_4$ (4.8 g/L). The glucose concentration was 6 g/L in all runs since it was previously found that higher concentrations did not have a positive effect on cell growth in shaken flasks (data not shown). Table 1 shows the nutritional factors studied and their corresponding levels. Table 2 presents the Plackett–Burman design trials. The 16 treatments were evaluated in triplicate.

Next, a 12-trial factorial design (2^3 with five central points) was carried out using the most significative nutritional factors of the screening design (peptone, yeast extract, and NaCl) to determine their influence on zeaxanthin and total carotenoid production. Table 3 shows the experimental conditions used. All media contained glucose (6 g/L), $CaCl_2$ (0.3 g/L), $MgSO_4.7H_2O$ (3.4 g/L), urea (0.5 g/L), and a micronutrient solution (1.3 mL/L). Strains were cultured in 1-L Erlenmeyer flasks with 300 mL of medium in an orbital shaker at 20°C and 200 rpm.

Because carotenoids are intracellular metabolites, the amount of carotenoids per volume of culture medium (i.e., the carotenoid concentration) is determined by the concentration of biomass in the culture media and the carotenoid amount per gram of dry biomass (i.e., the carotenoid content). To investigate the effect of each component in the carotenoid concentration, the responses studied were biomass concentration (g/L), zeaxanthin content (µg/g), zeaxanthin concentration (µg/L), total carotenoid content (µg/g), and total carotenoid concentration (µg/ L).

2.5 Zeaxanthin and total carotenoid production in a bioreactor

The media culture compositions that resulted in higher zeaxanthin and total carotenoid concentrations in the full factorial design were studied in a Biostat A Plus (Sartorius) bioreactor with 3 L of the medium at 20°C, pH 7, and airflow of 1 vvm. The agitation rate was regulated to ensure a minimum concentration of dissolved oxygen at 20% saturation, monitored with a dissolved oxygen sensor (Oxyferm FDA 325, Hamilton). Samples were taken every 12 h to monitor cell growth as explained in section 2.6, and carotenoid were extracted and analyzed as explained in section 2.2 and 2.6.

2.6 Analytical methods

Cell growth was determined by optical density at 600 nm with a Genesys 10S UV-vis (Thermo Scientific) spectrophotometer. Samples were diluted with distilled water to obtain absorbance values lower than 0.8 units. The relationship between optical density and dry cell weight was determined, and biomass concentrations were reported as grams of dry cells per liter of culture medium (g/L).

Glucose concentrations were determined by an HPLC (Shimadzu, Japan) equipped with an Aminex 87H column at 45°C and a refractive index detector (RID-10A). Ultrapure water was utilized in the mobile phase at a flow rate of 0.6 mL/min.

For carotenoid analysis and quantification, approximately 0.1 g of lyophilized cells were extracted with 1 mL of methanol (Merck). Supernatants were collected by centrifugation. The procedure was repeated three times until biomass bleaching occurred. The solvent was evaporated to dryness under a stream of nitrogen, and the dry extract was dissolved in 1 mL of acetone for chromatographic analysis. The chromatographic method used for carotenoid quantification has been previously described in detail (Delgado-Pelayo and Hornero-Méndez, 2012).

2.7 Statistical analyses

Analysis of variance (ANOVA) was performed via Info Stat/Estudiantil version 2019 software to determine the statistical significance of data differences ($p \leq 0.05$). Experimental designs were carried out using Statistica version 12 software (Statsoft Inc., USA).

3. Results

3.1 Effects of temperature on cell growth and zeaxanthin production

Table 4 presents the biomass concentration results, as well as zeaxanthin and total carotenoid content at 15, 20, 25, and 30ºC. Biomass concentrations were 1.4 ± 0.1 g/L in the experiments at 15°C and 20°C, decreasing to 1.1 ± 0.1 g/L at 25°C. At 30°C, there was no significant growth.

Zeaxanthin content in biomass was statistically equal at 15, 20, and 25°C, reaching a mean value of 205 μg/g. Total carotenoid content (zeaxanthin and its precursors β-cryptoxanthin and β-carotene) increased with temperature up to 383 ± 32 μg/g at 25 °C. Because biomass yield decreased at 25°C and zeaxanthin content remained constant with temperature, a temperature of 20°C was selected for further experiments.

3.2 Screening of nutrients

Plackett–Burman design-based experiments were conducted to identify nutritional components that enhance biomass growth in *Flavobacterium sp.* P8. Four nitrogen sources were tested (i.e., peptone, yeast extract, urea, and NH_4Cl). In addition, as the *Flavobacterium* strain was isolated from seawater, the salts studied were those present in commercial sea salt as the main components (i.e., Na_2HPO_4, NaCl, KCl, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, and $FeCl_3$). A micronutrient solution composed of salts at trace levels was also included as a unique factor. ~~The impact of the eleven factors studied was statistically analyzed using Plackett-Burman methodology~~. The results are shown in Tables 2 and 5. Yeast extract, peptone, NaCl, micronutrients, $CaCl_2$, $MgSO_4$, NaH_2PO_4, and urea had a positive effect on biomass growth. However, NH_4Cl, KCl, and $FeCl_3$ had no significant effect. Among the variables producing a positive effect, peptone, yeast extract, and NaCl contributed 85% of the biomass improvement. Therefore, these three medium components were selected for further optimization studies.

3.3 Design of a medium culture for zeaxanthin production

The amounts of yeast extract, peptone, and NaCl were increased to improve biomass concentrations and study their effects on carotenoid production. A 2^3+1 full factorial was carried out with the lowest levels set at the highest levels of the previous Plackett–Burman design. Table 3 shows the media composition for each run. The remaining factors with a positive effect on biomass production were set at the highest value in all runs. The experimental responses are shown in Table 3, and the statistical analysis is presented in Table 6. Biomass concentrations were positively affected by yeast extract and negatively by NaCl. The ~~significative~~ curvature ($p < 0.05$) suggests a maximum at the central point (runs 9-13, 7 g/L peptone, 7 g/L yeast extract, and 15 g/L NaCl). Then, initially, increments of yeast extract and NaCl favored biomass production. With higher levels of yeast extract, biomass remained elevated, but higher levels of NaCl (12 g/L) resulted in lower biomass concentrations.

Similarly, zeaxanthin and total carotenoid concentrations presented a maximum ~~significative curvature~~ ($p < 0.05$). Zeaxanthin concentrations were negatively affected by yeast extract and peptone but positively by NaCl, resulting in the ~~maximum~~ improvement of zeaxanthin concentration in ~~during~~ run 5 (2 g/L peptone, 2g/L yeast extract, and 24 g/L NaCl). Under these conditions, despite the low biomass yield, the zeaxanthin content was the highest, which resulted in the highest zeaxanthin concentration. Thus, this medium was selected for further studies in a bioreactor.

Run 2 (peptone 2 g/L, 12 g/L yeast extract, and 6 g/L of NaCl), run 5, and central point presented the highest total carotenoid concentration. Although lower zeaxanthin concentrations were obtained in runs 2 and the central point than in run 5, higher rates of oxygen mass transfer in the bioreactor could favor intermediate conversion to zeaxanthin. Then, considering the positive effect that NaCl had on zeaxanthin concentrations, medium composition at the central point (runs 9–13) were also evaluated in a bioreactor.

3.4 Zeaxanthin and total carotenoid production in a bioreactor

Zeaxanthin and total carotenoid production were studied in a 5 L-bioreactor with the two culture media selected previously (medium 5: 2 g/L peptone, 2g/L yeast extract, and 24 g/L NaCl; and the central point (CP) medium: 7 g/L peptone, 7 g/L yeast extract, and 15 g/L NaCl). The results are shown in Figure 1. With medium 5, a stationary growth phase was achieved after approximately 20 h, reaching a biomass concentration of 1.5 ± 0.1 g/L. Although glucose was almost totally consumed after approximately 48 h, the highest zeaxanthin and total carotenoid concentrations of 312 ± 12 µg/L and 439 ± 19 µg/L, respectively, were achieved after 72 h. Zeaxanthin and total carotenoid content in dry biomass resulted in 208 ± 7 µg/g and 293 ± 13 µg/g, respectively. The metabolic intermediates β-carotene and β-cryptoxanthin were detected, with β-carotene and zeaxanthin being the major carotenoids produced in the first 34 h. Then, zeaxanthin content reached 70% of the total carotenoids after 72 h.

After 20 h culture with CP medium, maximum biomass concentration was 5.8 ± 0.1 g/L with nearly complete consumption of glucose. The total carotenoid production increased to 2.18 ± 0.15 mg/L with an almost complete conversion of the intermediate compounds in zeaxanthin (2.15 ± 0.15 mg/L, 98% of zeaxanthin). Zeaxanthin and total carotenoid in dry mass achieved 370 ± 12 µg/g and 377 ± 13 µg/g, respectively.

4. Discussion

Flavobacterium species are a well-known natural source of carotenoids. The amount and main carotenoids produced by different strains may depend on fermentation conditions, such as temperature, media composition, stirring rate, and aeration, among others (Zhang et al., 2018). In this work, zeaxanthin content in biomass was similar at 15, 20, and 25°C, but β-carotene and β-cryptoxanthin accumulation increased with temperature, resulting in a rise of total carotenoid content. Temperature influences the biosynthetic pathways of microorganisms and may affect the carotenoid biosynthesis modifying the content and activity of the enzymes involved in carotenoid production, thus affecting their synthesis (Hayman et al., 1974). Therefore, an increase in these intermediates could be associated with a rise in enzyme activity or concentration due to temperature increments (Britton et al., 1977). Other factor that could contribute was the increment in active oxygen species in bacteria as temperature increases, which stimulates carotenogenesis (Mandelli et al., 2012).

The development of a suitable medium culture to produce zeaxanthin is needed to improve yields and increase the economic viability of the bioprocess. Through Placket-Burman statistical analysis, the studied variables were reduced to peptone, yeast extract and NaCl. Further modifications to the medium composition resulted in the highest zeaxanthin content in shaken flasks ~~(105 ± 7 µg/g)~~ and total carotenoid content (~~158 ± 10 µg/g),~~ which corresponds to run 5 (2 g/L peptone, 2g/L yeast extract, and 24 g/L NaCl~~),~~ although the biomass concentration was only 2.6 ± 0.1 g/L. As the concentrations of peptone and yeast extract were the lowest

within the experimental design, biomass production could have been limited by a lack of nutrients. The biomass concentrations were higher in runs with more nutrients, but the content of carotenoids in biomass were ~~was even higher~~ lower ~~than in runs with higher concentrations of biomass~~ Therefore, run 5 was the medium with the highest zeaxanthin concentration of ~~272 ± 19 µg/L~~, but the total carotenoid concentration was similar to those obtained in runs 2 ~~(peptone 2 g/L, 12 g/L yeast extract, and 6 g/L of NaCl)~~ and 9–13 ~~(7 g/L peptone, 7g/L yeast extract, and 15 g/L NaCl).~~ Because *Flavobacterium* species are described as mostly aerobic (Bernardet and Bowman, 2006), higher amounts of biomass could have demanded more oxygen for growth and the viability and maintenance of cells, resulting in a lower effective oxygen concentration needed for the sequential conversion of β-carotene and β-cryptoxanthin into zeaxanthin. This fact could explain the lower levels of zeaxanthin concentration in runs 2 ~~(peptone 2 g/L, 12 g/L yeast extract and 6 g/L of NaCl)~~ and 9–13 ~~(7 g/L peptone, 7g/L yeast extract and 15 g/L NaCl)~~ and the relatively higher accumulation of intermediates in shaken flasks since both presented relatively high biomass concentrations ~~(4.1 and 4.5 g/L respectively~~).

When *Flavobacterium* sp. P8 was cultivated in a bioreactor with CP medium (7 g/L peptone, 7g/L yeast extract, and 15 g/L NaCl), biomass and total carotenoid concentrations were higher than those obtained in shaken flasks, ~~reaching 5.8 ± 0.1 g/L and 2.15 ± 0.15 mg/L, respectively~~. β-carotene and β-cryptoxanthin concentrations remained low and practically constant for 72 h, and zeaxanthin represented 98% of the total carotenoid concentration. These results show a 4.5-fold improvement in total carotenoid concentrations and 9.2-fold improvement in zeaxanthin concentrations when compared to the values obtained under shaken flask conditions.

However, when cultured in a bioreactor with medium 5 (2 g/L peptone, 2g/L yeast extract, and 24 g/L NaCl), zeaxanthin and total carotenoid concentrations were similar to the results found for the shaken flask condition ~~(0.312 ± 0.012 mg/L and 0.27 ± 0.02 mg/L, respectively)~~, but lower biomass concentrations were produced. Therefore, the carotenoid production limitation in this medium could be associated with nutritional factors other than oxygen.

When scaling up to a bioreactor, agitation and aeration control can increase the availability of oxygen due to improvements in its mass transfer coefficient (Masetto et al., 2001; Valduga et al., 2009). Differences in cell growth, substrate consumption, and carotenoid production in shaken flasks and bioreactors have been reported for *Sporidiobulous salmnicolor* (Valduga et al., 2011). These differences highlight the limitation of shaken flask optimizations when working with aerobic bacteria and oxygen dependent products.

Several zeaxanthin-producing strains with different culture conditions have been reported (Table 7). Although *Flavobacterium* sp. P8's total carotenoid concentrations were among the lowest values, its performance improved in a bioreactor, indicating that it has potential as an alternative zeaxanthin source, and further optimization must include oxygen and other nutrients.

5. Conclusions

A medium was formulated for the production of zeaxanthin with an Antarctic *Flavobacterium* strain. In the optimized medium, the strain was able to produce 2.15 ± 0.15 mg/L of zeaxanthin in a bioreactor, which represented 98% of the total carotenoids produced. Peptone, yeast extract, NaCl, and aeration played a fundamental role in enhancing carotenoid production and the conversion of intermediates to zeaxanthin. This study shows that *Flavobacterium sp* P8 is a promising source of zeaxanthin ~~production~~.

Credit author statement

Eugenia Vila: conceptualization, methodology, formal analysis, writing - original draft, project administration, funding acquisition.

Dámaso Hornero-Mendez: validation, writing - review & editing.

Claudia Lareo: writing - review & editing, supervision.

Verónica Saraiva: conceptualization, methodology, formal analysis, writing - review & editing, supervision, funding acquisition.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflict of Interest

No conflict of interest declared.

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References

Bernardet, J.-F., Bowman, J.P., 2006. The Genus Flavobacterium, in: The Prokaryotes. Springer New York, New York, NY, pp. 481–531. https://doi.org/10.1007/0-387-30747-8_17

Bhosale, P., Larson, A.J., Bernstein, P.S., 2004. Factorial analysis of tricarboxylic acid cycle intermediates for optimization of zeaxanthin production from Flavobacterium multivorum. J. Appl. Microbiol. 96, 623–629. https://doi.org/10.1111/j.1365-2672.2004.02197.x

Britton, George, 1995. Structure and properties of carotenoids in relation to function. FASEB J. 9, 1551–1558.

Britton, G, 1995. Structure and properties of carotenoids in relation to function. FASEB J. 9, 1551–8.

Britton, G., Brown, D.J., Goodwin, T.W., Leuenberger, F.J., Schocher, A.J., 1977. The carotenoids of Flavobacterium strain R1560. Arch. Microbiol. 113, 33–7.

Delgado-Pelayo, R., Hornero-Méndez, D., 2012. Identification and Quantitative Analysis of Carotenoids and Their Esters from Sarsaparilla (Smilax aspera L.) Berries. J. Agric. Food Chem. 60, 8225–8232. https://doi.org/10.1021/jf302719g

Hayman, E.P., Yokoyama, H., Chichester, C.O., Simpson, K.L., 1974. Carotenoid biosynthesis

in Rhodotorula glutinis. J. Bacteriol. 120, 1339–43.

Krinsky, N.I., Landrum, J.T., Bone, R.A., 2003. Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. Annu. Rev. Nutr. 23, 171–201. https://doi.org/10.1146/annurev.nutr.23.011702.073307

Liu, Y.S., Wu, J.Y., 2007. Optimization of cell growth and carotenoid production of Xanthophyllomyces dendrorhous through statistical experiment design. Biochem. Eng. J. 36, 182–189. https://doi.org/10.1016/j.bej.2007.02.014

Mandelli, F., Miranda, V.S., Rodrigues, E., Mercadante, A.Z., 2012. Identification of carotenoids with high antioxidant capacity produced by extremophile microorganisms. World J. Microbiol. Biotechnol. 28, 1781–1790. https://doi.org/10.1007/s11274-011-0993-y

Masetto, A., Flores-Cotera, L.B., Díaz, C., Langley, E., Sanchez, S., 2001. Application of a complete factorial design for the production of zeaxanthin by Flavobacterium sp. J. Biosci. Bioeng. 92, 55–8.

Navarrete-Bolaños, J.L., Rangel-Cruz, C.L., Jiménez-Islas, H., Botello-Alvarez, E., Rico-Martínez, R., 2005. Pre-treatment effects on the extraction efficiency of xanthophylls from marigold flower (Tagetes erecta) using hexane. Food Res. Int. 38, 159–165. https://doi.org/10.1016/j.foodres.2004.09.007

Sajilata, M.G., Singhal, R.S., Kamat, M.Y., 2008. The Carotenoid Pigment Zeaxanthin—A Review. Compr. Rev. Food Sci. Food Saf. 7, 29–49. https://doi.org/10.1111/j.1541-4337.2007.00028.x

Shin, H.S., Kim, J.W., Kim, J.H., Lee, D.G., Lee, S., Kil, D.Y., 2016. Effect of feeding duration of diets containing corn distillers dried grains with solubles on productive performance, egg quality, and lutein and zeaxanthin concentrations of egg yolk in laying hens. Poult. Sci. 95, 2366–2371. https://doi.org/10.3382/ps/pew127

Valduga, E., Schwartz, C.R.M., Tatsch, P.O., Tiggemann, L., Di Luccio, M., Treichel, H., 2011. Evaluation of aeration and substrate concentration on the production of carotenoids by Sporidiobolus salmonicolor (CBS 2636) in bioreactor. Eur. Food Res. Technol. 232, 453–462. https://doi.org/10.1007/s00217-010-1410-8

Valduga, E., Valério, A., Treichel, H., Júnior, A.F., Di, M.L., 2009. Optimization of the production of total carotenoids by Sporidiobolus salmonicolor (CBS 2636) using response surface technique. Food Bioprocess Technol. 2, 415–421. https://doi.org/10.1007/s11947-008-0066-x

Vila, E., Hornero-Méndez, D., Azziz, G., Lareo, C., Saravia, V., 2019. Carotenoids from heterotrophic bacteria isolated from Fildes Peninsula, King George Island, Antarctica. Biotechnol. Reports 21. https://doi.org/10.1016/j.btre.2019.e00306

Zhang, Y., Liu, Z., Sun, J., Xue, C., Mao, X., 2018. Biotechnological production of zeaxanthin

by microorganisms. Trends Food Sci. Technol. 71, 225–234.
https://doi.org/10.1016/j.tifs.2017.11.006

Table 1. Nutritional factors' concentrations at different levels in the Placket-Burman design.

Code	Factors	Lower level (-) (g/L)	Higher level (+) (g/L)
$\mathbf{X_1}$	Peptone	0.2	2.0
$\mathbf{X_2}$	Yeast extract	0.2	2.0
$\mathbf{X_3}$	Urea	0.05	0.5
$\mathbf{X_4}$	NH_4Cl	0.08	0.8
$\mathbf{X_5}$	$Na_2HPO_4 \cdot 7H_2O$	0	6.3
$\mathbf{X_6}$	NaCl	0	0.2
$\mathbf{X_7}$	KCl	0	3.4
$\mathbf{X_8}$	$MgSO_4 \cdot 7H_2O$	0	0.3
$\mathbf{X_9}$	$CaCl_2$	0	0.03
$\mathbf{X_{10}}$	$FeCl_3 \cdot 6H_2O$	0	0.04
$\mathbf{X_{11}}$	Micronutrients solution	0	400 µL

Micronutrient solution: H_3BO_3 (12.8 g/L), $LiSO_4$ (1.0 g/L), $MnSO_4$ (3.2 g/L), $CoCl_2$ (2.0 g/L), $CuSO_4 \cdot H_2O$ (4.0 g/L), $NiSO_4$ (2.5 g/L), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (2.8 g/L), ZnSO4 (4.8 g/L)

Table 2. Plackett–Burman design for eleven variables ($X_1 – X_{11}$) and 4 dummy variables (X_{12} - X_{15}) coded to complete the 16-trail run design.

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	Biomass (g/L)	Glucose consumption (g/L)
1	-1	-1	-1	-1	1	1	1	1	1	1	-1	-1	-1	-1	1	0.5	0.7
2	-1	-1	-1	1	1	1	-1	1	-1	-1	-1	1	1	1	-1	0.2	0.3
3	-1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	1	-1	0.3	0.3
4	-1	-1	1	1	1	-1	-1	-1	-1	1	1	1	-1	-1	1	0.2	0.2
5	-1	1	-1	-1	-1	1	1	-1	-1	1	1	1	-1	1	-1	1.3	2.8
6	-1	1	-1	1	-1	1	-1	-1	1	-1	1	-1	1	-1	1	1.0	2.2
7	-1	1	1	-1	-1	-1	1	1	-1	-1	-1	1	1	-1	1	1.1	1.9
8	-1	1	1	1	-1	-1	-1	1	1	1	-1	-1	-1	1	-1	0.8	1.3
9	1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	-1	-1	0.6	1.0
10	1	-1	-1	1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	0.5	1.1
11	1	-1	1	-1	-1	1	-1	-1	1	-1	-1	1	-1	1	1	0.5	1.1
12	1	-1	1	1	-1	1	1	-1	-1	1	-1	-1	1	-1	-1	0.5	0.9
13	1	1	-1	-1	1	-1	-1	-1	-1	1	-1	-1	1	1	1	1.2	1.8
14	1	1	-1	1	1	-1	1	-1	1	-1	-1	1	-1	-1	-1	1.3	1.9
15	1	1	1	-1	1	1	-1	1	-1	-1	1	-1	-1	-1	-1	1.8	3.6
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2.1	4.4

Table 3. Full factorial design results (biomass, zeaxanthin, and total carotenoid responses after 48 h of fermentation).

Run	Factor (g/L)			Content (μg/$g_{biomass}$)		Concentration (μg/L)		Biomass (g/L)
	NaCl	Peptone	Yeast extract	Zeaxanthin	Total carotenoid	Zeaxanthin	Total carotenoid	
1	6	2	2	46 ± 3	89 ± 6	111 ± 6	212 ± 13	2.4 ± 0.1
2	6	2	12	30 ± 1	103 ± 2	122 ± 3	422 ± 6	4.1 ± 0.1
3	6	12	2	26 ± 2	70 ± 5	79 ± 7	203 ±15	3.1 ± 0.1
4	6	12	12	18 ± 1	27 ± 1	77 ± 1	113 ± 1	4.2 ± 0.1
5	24	2	2	105 ± 7	158 ± 10	272 ± 19	411 ± 27	2.6 ± 0.1
6	24	2	12	40 ± 3	81 ± 4	156 ± 10	315 ± 17	3.9 ± 0.1
7	24	12	2	34 ± 2	63 ± 4	85 ± 6	157 ± 10	2. 5± 0.1
8	24	12	12	16 ± 1	16 ± 1	53 ± 1	53 ± 1	3.4 ± 0.1
9	15	7	7	50 ± 2	101 ± 4	213 ± 9	434 ± 17	4.3 ± 0.1
10	15	7	7	44 ± 1	84 ± 2	197 ± 2	379 ± 9	4.5 ± 0.1
11	15	7	7	46 ± 2	93 ± 4	212 ± 9	428 ± 18	4.6 ± 0.1
12	15	7	7	47 ± 2	100 ± 3	216 ± 7	459 ±12	4.6 ± 0.1
13	15	7	7	47 ± 2	94 ± 3	218 ± 7	430 ±12	4.6 ± 0.1

Table 4. Biomass concentrations and carotenoid content in dry biomass at different temperatures in shaken flasks at 200 rpm and 12 h of fermentation.

Temperature (°C)	Biomass (g/L)	Zeaxantin (µg/g)	β-cryptoxanthin (µg/g)	β-carotene (µg/g)	Total carotenoid (µg/g)
15	1.4 ± 0.1	217 ± 12	5 ± 2	nd	223 ± 8
20	1.4 ± 0.1	173 ± 24	33 ± 6	nd	206 ± 34
25	1.1 ± 0.1	223 ± 18	81 ± 2	79 ± 14	383 ± 32

Table 5. The effects of factors, including *p*-values, confidence intervals, and the sum of squares (SS) percentages for biomass production in the Plackett-Burman design.

Variable	Effect	-% Confidence limit	+% Confidence limit	*p*-value	SS%
Peptone	0.417	0.343	0.491	0.000	14.6
Yeast Extract	0.883	0.809	0.957	0.000	65.5
Urea	0.075	0.001	0.149	0.047	0.5
NH_4Cl	-0.067	-0.141	0.007	0.076	0.4
NaH_2PO_4	0.125	0.051	0.199	0.002	1.3
NaCl	0.250	0.176	0.324	0.000	5.2
$CaCl_2$	0.175	0.101	0.249	0.000	2.6
$MgSO_4$	0.167	0.093	0.241	0.000	2.3
KCl	0.008	-0.066	0.082	0.820	0.0
$FeCl_3$	0.067	-0.007	0.141	0.076	0.4
Micro	0.192	0.118	0.266	0.000	3.1
Dummy 1	0.067	-0.007	0.141	0.076	0.4
Dummy 2	-0.008	-0.082	0.066	0.820	0.0
Dummy 3	0.008	-0.066	0.082	0.820	0.0
Dummy 4	0.050	-0.024	0.124	0.178	0.2

$R^2 = 0.9646$, (95% significant level)
The *p*-values less than 0.05 denoted the significant factors.

Table 6. The effects of factors and *p*-values for biomass, zeaxanthin, and total carotenoid concentrations in the 2^3 full-factorial design.

Response	**Biomass (g/L)**		**Zeaxanthin concentration (µg/L)**		**Total carotenoid concentration (µg/L)**	
Factor	**Effect**	***p* - value**	**Effect**	***p* - value**	**Effect**	***p* - value**
Mean	3.275	0.000	119.363	0.000	235.675	0.000
Curvature	2.490	0.000	184.155	0.000	380.930	0.001
NaCl (1)	-0.350	0.009	44.125	0.014	-4.000	0.919
Peptone (2)	0.050	0.583	-91.825	0.000	-209.700	0.003
Y. extract (3)	1.250	0.000	-34.975	0.022	-20.250	0.613
1 by 2	-0.350	0.009	-53.225	0.007	-49.250	0.247
1 by 3	-0.150	0.134	-39.275	0.022	-79.900	0.087
2 by 3	-0.250	0.033	17.375	0.208	-77.100	0.095
R^2	0.991		0.976		0.941	

The *p*-values less than 0.05 denoted the significant factors.

Table 7. Fermentation parameters of different zeaxanthin-producing strains.

Microorganism	Fermentation conditions	Time (h)	Biomass (g /L)	Zeaxanthin content (mg/g)	Zeaxanthin concentration (mg/L)	Total carotenoids (mg /L)	Reference
Shaken Flasks							
Flavobacterium sp. P8	Medium 5, 20ºC, 200 rpm	48	2.6 ± 0.1	0.105 ± 0.007	0.27 ± 0.02	0.41 ± 0.03	This work
Flavobacterium sp. P8	CP medium, 20ºC, 200 rpm	48	4.5 ± 0.1	0.047 ± 0.002	0.21 ± 0.09	0.43 ± 0.02	This work
Flavobacterium multivorum ATCC 55238	30ºC, 250 rpm, TCA intermediates optimized	44	6.5	-	9.8 ± 0.2	10.7 ± 0.6	Bhosale *et al.* 2004
Flavobacterium sp. (ATCC 25582)	1% yeast extract, 29ºC, 180 rpm	48	-	-	0.75 ± 0.03	-	Alcantara and Sánchez 1999
Formosa sp. KMW	Marine broth + glucose, 37ºC, 200 rpm	120	4.5 ± 0.7	0.166 ± 0.013	-	0.66 ±0.06	Sowmya and Sachindra 2015
Muricada sp. YUAB-SO-45	Marine broth + glutamic acid, 30ºC, 125 rpm	72	2.1	1.18 ± 0.10	2.16	-	Prabhu *et al*. 2013
Muricada sp. YUAB-SO-11	Marine broth + glutamic acid, 30ºC, 125 rpm	48	2.6	1.47 ± 0.02	3.14	-	Prabhu *et al*. 2013
Paracoccus zeaxanthinifaciens ATCC 21588	30ºC, 180 rpm	72	-	-	11.63	-	Joshi and Singhal 2016
Spingomonas natatoria KODA19-6	Modified Zobell broth, 30ºC, 120 rpm	96	0.4	4.1	0.62	-	Thawornwiriyanun *et al*. 2012
Bioreactor							
Flavobacterium sp. P8	Medium 5, 20ºC, 200 rpm, 20%pO_2	72	1.5 ± 0.1	0.208 ± 0.007	0.312 ± 0.012	0.411 ± 0.027	This work
Flavobacterium sp. P8	CP medium, 20ºC, 200 rpm, 20%pO_2	72	5.8 ± 0.1	0.37 ± 0.01	2.15 ± 0.15	2.18 ± 0.15	This work
Flavobacterium sp. (ATCC 21588)	4.6% corn steep liquor, 600 rpm, 2 vvm	72	-	-	9.8	11.4	Masetto *et al.* 2001
Muricada lutaonensis CC-HSB-11^T	Marine broth, 40ºC, 15% pO_2, pH 7	72	1.17 ± 0.13	-	3.12 ± 0.18	-	Hameed *et al.* 2011

Medium 5: 24 g/L NaCl, 2 g/L yeast extract, 2 g/L peptone; CP medium: 15 g/L NaCl, 7 g/L yeast extract, 7 g/L peptone; TCA: malic acid (6.02 mM), isocitric acid (6.20 mM) and α-ketoglutarate (0.02 mM).

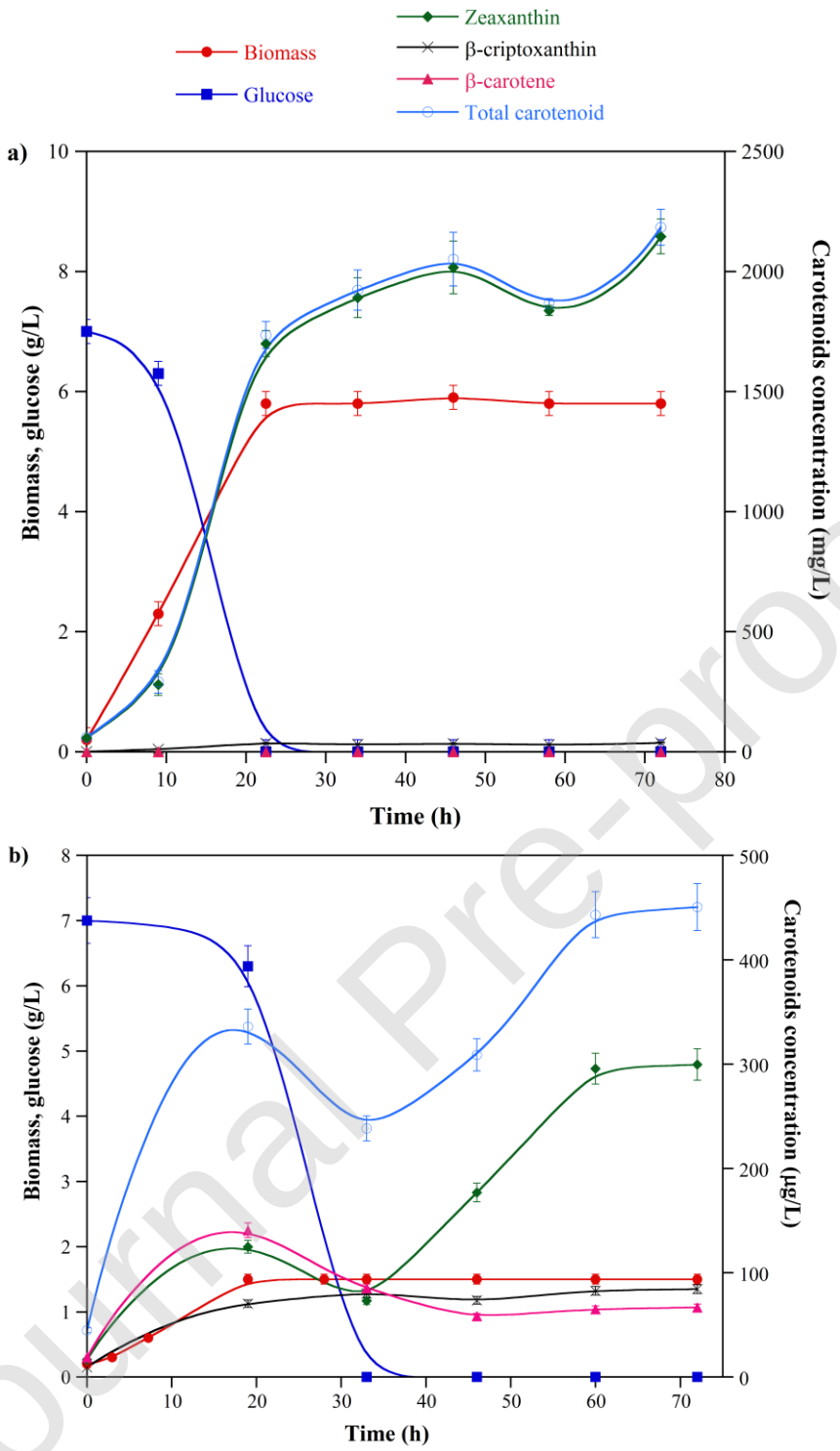

Figure 1. Biomass and carotenoids profiles in bioreactor at 20ºC, 20%pO_2. (A) CP medium: 7 g/L peptone, 7 g/L yeast extract and 15 g/L NaCl; (B) run 5 medium: 2 g/L peptone, 2 g/L yeast extract and 24 g/L NaCl.